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In vivo characterization of human *APOA5* haplotypes

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Abstract

Increased plasma triglyceride concentrations are an independent risk factor for cardiovascular disease. Numerous studies support a reproducible genetic association between two minor haplotypes in the human apolipoprotein A5 gene (*APOA5*) and increased plasma triglyceride concentrations. We thus sought to investigate the effects of these minor haplotypes (*APOA5*2* and *APOA5*3*) on ApoAV plasma levels through the precise insertion of single-copy *APOA5* haplotypes at a targeted location (*Hprt*) in the mouse genome. While we found no difference in the amount of human plasma ApoAV in mice containing the common *APOA5*1* or minor *APOA5*2* haplotype, the introduction of the single *APOA5*3*-defining allele (19W) resulted in three fold lower ApoAV plasma levels, consistent with existing genetic association studies. These results indicate that the S19W polymorphism is likely to be functional and explain the strong association of this variant with plasma triglycerides, supporting the value of sensitive in vivo assays to define the functional nature of human haplotypes.

Keywords: *APOA5*; Triglycerides; Cardiovascular disease; Haplotypes; Genetically engineered mice

Elevated lipid plasma levels are a significant risk factor for stroke and coronary artery disease [1,2]. The apolipoprotein gene cluster (*APOA5/APOA4/APOC3/APOA1*) on human chromosome 11 (Fig. 1A) is one locus that has been genetically linked to interindividual differences in plasma lipid levels [3,4]. Specifically, *APOA5* is one member of this cluster that has been strongly implicated in influencing plasma triglyceride levels in both mice and humans [5]. Mice lacking *Apoa5* have increased plasma triglyceride concentrations, while mice overexpressing human *APOA5* exhibit decreased plasma triglyceride concentrations [5]. In humans, numerous studies in several different ethnic populations have shown significant association between two minor *APOA5* haplotypes, *APOA5*2* and *APOA5*3,* and elevated plasma triglyceride levels [5–12]. It is estimated that 53% of Hispanics, 35% of African-Americans, and 24% of Caucasians carry at least one of these two minor haplotypes [6], thus suggesting these haplotypes may be a common risk factor for atherosclerosis in humans.

Recent in vitro studies have provided initial clues to the possible functional alleles within these minor *APOA5* haplotypes. In humans, genetic studies showed that carriers of the minor *APOA5*2* haplotype [defined by the following nucleotide changes: −1131T→C (rs662799), −3A→G (rs651821), 751G→T (rs2072560), and 1891T→C (rs2266788)] displayed significantly lower ApoAV levels compared to the common −1131T variant (152.4 ng/ml versus 200.8 ng/ml) as well as elevated triglyceride levels (1.46 mmol/L versus 0.88 mmol/L) [13]. However, subsequent in vitro functional studies of −1131C and other *APOA5*2* variants yielded no significant alteration in transcription or translation in cell lines [14]. One possible explanation for this observation is based on the strong linkage disequilibrium of the *APOA5*2* haplotype with three neighboring *APOC3* variants [15] (−482C→T, −455T→C,

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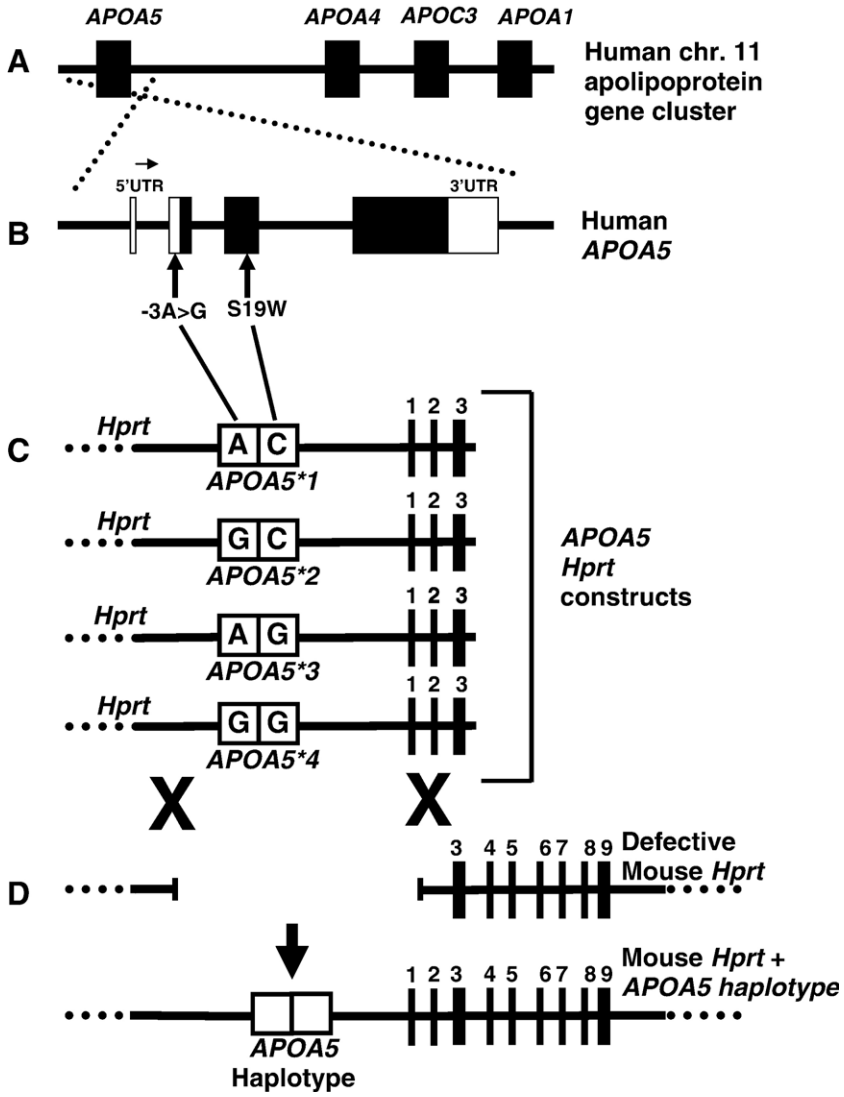

Fig. 1. Generation of *APOA5* haplotype mouse lines. (A) Apolipoprotein gene cluster on human chromosome 11. (B) *APOA5* gene and locations of haplotype-determining variants. (C) *APOA5* haplotype constructs. (D) Recombination of the *APOA5* haplotype constructs into a defective *Hprt* locus in the mouse.

and 3238G→C) that have been repeatedly associated with elevated plasma triglyceride concentrations in several human populations [7,16–30]. Based on all these results, it remains unclear whether the *APOA5*2* haplotype contributes to elevated plasma triglycerides through a cooperative effect of all four *APOA5* variants or in association with linked causal *APOC3* variants.

In contrast, the *APOA5*3* haplotype associated with elevated triglyceride concentrations is defined by a single coding variant allele (rs3135506, changing 19S to 19W) and in vitro studies revealed a functional consequence of this resulting amino acid substitution [14]. Specifically, molecular modeling of the W19 variant predicted reduced translocation across the endoplasmic reticulum, which was functionally supported through in vitro studies indicating a two fold reduction in protein secretion in comparison to the common 19S allele at this position [14]. In addition, unlike *APOA5*2, APOA5*3* showed no association with any neighboring *APOC3* SNP allele [15], suggesting that it is an isolated genetic variant that is functionally responsible for the *APOA5*3* haplotype association with elevated triglyceride levels.

To assess further whether minor human *APOA5* haplotypes by themselves result in altered ApoAV plasma levels in vivo, we inserted *APOA5* haplotype constructs into mice through targeted embryonic stem (ES) cell engineering. To circumvent the standard mouse transgenic complications of variation in copy number and integration sites, we inserted each haplotype construct into a vector designed to rescue an ES cell line harboring a partially defective hypoxanthine phosphoribosyltransferase gene (*Hprt*), thereby allowing for appropriately targeted colonies to be positively selected using hypoxanthine aminopterin thymidine (HAT) medium [31]. Through these studies, we find that, while *APOA5*2* failed to result in any considerable change in ApoAV plasma levels compared to *APOA5*1,* introduction of the W19 variant into the minor *APOA5*2* haplotype significantly reduced ApoAV plasma levels in vivo.

Results

Generation of APOA5 haplotype mouse lines

To generate each *APOA5* haplotype construct, we electronically identified two publicly available bacterial artificial chromosome (BAC) clones that corresponded to haplotypes *APOA5*1* (BAC CTC-270C21) and *APOA5*2* (BAC RP11-442E11). From these BACs, we cloned an 11,360-bp fragment (chromosome 11: 116,163,727–116,175,086; hg18) that corresponds to the human *APOA5* region (Fig. 1B; see Materials and methods). The minor *APOA5*3* haplotype was then constructed through site-directed mutagenesis of the cloned *APOA5*1* haplotype, creating a single change of S19 to 19W. In addition, to ascertain the consequence of the 19W change we generated a fourth hybrid haplotype of *APOA5*2* and *APOA5*3* through similar site-directed mutagenesis of cloned haplotype *APOA5*2,* changing c56C to c56G, hereafter referred to as *APOA*4* (Fig. 1C).

All four haplotypes were cloned into an *Hprt* replacement vector [31] and each construct was independently electroporated into a previously described E14TG2a *Hprt*-deficient 129 ES cell line (Fig. 1D) (see Materials and methods) [32]. Colonies resistant to HAT medium were screened using PCR and Southern analysis (data not shown) to confirm proper targeting and single integration events at the *Hprt* locus and were subsequently injected into C57BL/6J blastocyst stage embryos. The resulting male chimeras were bred to C57BL/6J females to allow for detection of ES cell transmission based on the 129 strain cell line-derived agouti coat color marker. Female agouti mice positive for the transgene were bred to C57BL/6J males to generate transgene-positive hemizygous males (*Hprt* is located on the X chromosome). While haplotypes *APOA5*1, APOA5*2,* and *APOA5*4* were successfully transmitted through the germ line, none of the resulting chimeric mice from two separate *APOA5*3* ES clones contained that haplotype, despite repeated attempts. We thus limited our analysis to the aforementioned mouse lines.

*Plasma ApoAV levels are reduced for the haplotype APOA5*4 but not the APOA5*2 mouse line*

To date, *APOA5* expression has been detected only in the liver, from which it is secreted into the plasma, functioning in increasing lipolysis in the periphery as well as LDL clearance by the liver. We thus sought to determine if ApoAV plasma levels were altered due to the allelic variants found within their respective haplotypes. We measured human ApoAV plasma levels in male mice using a human-specific ApoAV antibody. As a control, we found no detectable levels of human ApoAV in littermates that were negative for the transgene ($n=18$). In contrast, we detected an average ApoAV plasma level of 0.628 mg/dl in transgenic mice for the most common human *APOA5*1* haplotype (Fig. 2A). It is worth noting that these levels are considerably lower than the average human levels that are estimated to be 17.92±7.48 mg/dl [13]. This is likely the result of the single-copy nature of the transgene as well as the

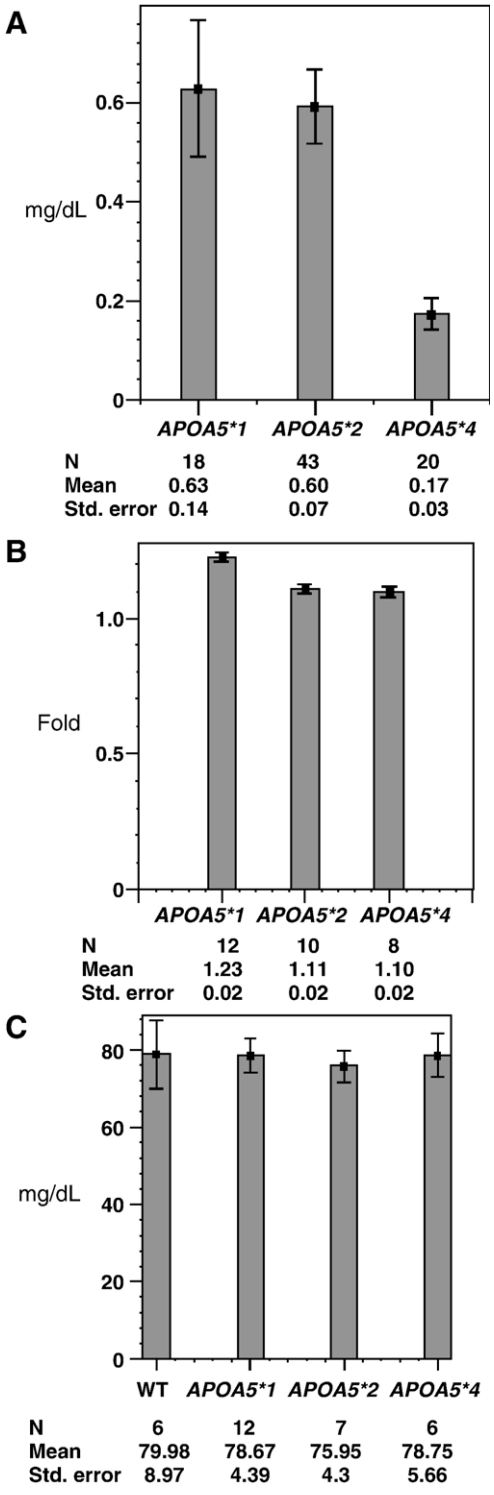

Fig. 2. *APOA5* protein, mRNA and triglyceride levels in *APOA5* haplotype mice. (A) Human ApoAV plasma levels. (B) Human *APOA5* liver mRNA levels normalized to 18S RNA expression. (C) Total plasma triglyceride levels. Error bars for all three charts indicate the standard error; *N* below the chart indicates the number of mice assayed, followed by the mean ApoAV or triglyceride plasma levels (mg/dl) and fold for mRNA levels and the standard error in each experiment.

genomic environment of the *Hprt* locus. We next determined if the minor *APOA5* haplotypes associated with increased triglyceride levels in humans displayed altered ApoAV plasma levels compared to *APOA5*1.* No significant difference (0.63 versus 0.60 mg/dl; $p=0.810$, *t* test) was observed between *APOA5*1* and *APOA5*2* transgenic mice. In contrast, *APOA5*4,* which contains a single S19 to W19 change within *APOA5*2,* showed significantly lower ApoAV plasma levels compared to *APOA5*1* (0.17 versus 0.63 mg/dl; $p=0.002$, *t* test) (Fig. 2A). Further studies of mRNA levels in liver tissue from these three mouse lines revealed minimal differences in *APOA5* transcript levels, indicating that the decreased ApoAV protein in *APOA5*4* transgenic mice occurs posttranscriptionally (Fig. 2B). Combined, these results indicate that while the *APOA5*2* variants do not have a significant effect on ApoAV plasma levels, the W19 allele alone resulted in substantially lower ApoAV levels.

Finally, we measured triglyceride levels in mice derived from all *APOA5* haplotype lines, observing no differences between these cohorts (Fig. 2C). This was not unexpected since the endogenous mouse locus for *Apoa5* remains intact within these *Hprt*-based targeted mice. Future studies aimed at determining the effects of these transgenes with *Apoa5*-deficient mice would assess directly the ability of these haplotypes to recapitulate the triglyceride phenotypic differences found in humans. Nevertheless, the established role of decreased ApoAV function leading to increased triglycerides in knockout mice is consistent with human *APOA5* minor haplotypes leading to lower ApoAV protein levels, which in turn raise triglycerides in plasma.

Discussion

Our goal in this study was to determine the in vivo effects of *APOA5* polymorphisms repeatedly associated with interindividual differences in plasma triglyceride concentrations. Using an *Hprt* mouse targeting scheme, we created male mice, each carrying a different single copy of a human *APOA5* haplotype. We were thus able to avoid the complexities of regular transgenic mice in which multiple copies and different insertion sites can significantly affect the expression of a transgene. In addition, this transgenic scheme enabled the disconnection of *APOA5* from the neighboring *APOC3* (as well as *APOA4* and *APOA1*), consequently revealing that only one of the two minor haplotypes associated with increase triglycerides in humans appears causative of this plasma phenotypic difference across humans.

Previous in vitro functional studies on the majority of variants defining *APOA5*2* have suggested that the variants within this haplotype are not functional [14]. However, these studies were conducted within cell lines and were not able to assess all haplotype-defining alleles in combination, but rather studied each independently based on predictions on how they may alter gene function. By inserting the entire *APOA5*2* haplotype into the mouse, we find that these alleles combined do not have a cooperative effect that might otherwise support their causal role in the observed human genetic association with increased triglycerides. These findings further suggest that this *APOA5*2* haplotype association may be explained due to its strong linkage disequilibrium with previously characterized triglyceride-elevating *APOC3* alleles. It will be interesting in future studies to determine the effects of *APOC3* minor haplotypes associated with increase plasma triglycerides levels through similar in vivo studies.

We can strongly infer that the S19W variant is functional. This is based on the observation that the single change of *APOA5*2* haplotype S19 to W19 resulted in a threefold decrease in ApoAV plasma levels in vivo. This decrease is consistent with previous functional studies that correlate this change with a significant reduction in ApoAV secretion levels in vitro [14]. The phenotypic outcome of such a reduction, elevated plasma triglyceride levels, can be inferred from *Apoa5* knockout mice. *Apoa5*-null homozygous mice have four times higher plasma triglyceride levels than their wild-type littermates [5], thus indicating that the expected phenotype of lower ApoAV would be higher plasma triglyceride levels. In conclusion, these results further support that individuals carrying the *APOA5*3* haplotype have a higher risk for elevated plasma triglyceride levels directly through the S19W genetic variant that defines this haplotype.

While this study was focused on *APOA5*-specific haplotypes, the results are relevant to the increasing number of genetic associations being identified in humans. These statistical associations gain power through validation in independent cohorts, but ultimately lack formal proof of their causative nature. Through a combination of in vitro and in vivo studies as described here it is anticipated that haplotypes can be functionally linked to their associated human phenotypes and provide an experimental means to define the ultimate functional allele(s) within these stretches of human DNA.

Materials and methods

Cloning the APOA5 Hprt constructs

BACs 270C21 (*APOA*1;* GenBank AP001481) and 442e11 (*APOA*2;* GenBank AC007707) were restriction digested sequentially with *Not*I and *Bst*EII and separated on a pulsed-field gel without ethidium bromide. Electronically predicted 11.5-kb fragments were gel excised and purified using a QiaEx II kit (Qiagen), blunt-end repaired using an EpiCenter CopyControl PCR cloning kit (Epicentre), and ligated into a pCCI vector (Epicentre). Five-kilobase *Hin*dIII fragments from both the *APOA5*1* and the *APOA5*2* clones in pCCI were cloned into a pBS vector (Invitrogen) to facilitate the ease of site-directed mutagenesis. In both vectors site-directed mutagenesis of the *APOA5*3* variant (c56C→G) was carried out using a Quickchange site-directed mutagenesis kit (Stratagene). After mutagenesis clones were reverified by sequencing with primers F1-AGAGGCCTCAGCTTTTCCAGGA, R1-GCGGATCCGAGCAGAGCAGA, R2-GCGGATCCGAGCAGAGCAGG, R3-TCTGGCTCTTCTTTCAGCGTTTTC, and R4-TCTGGCTCTTCTTTCAGCGTTTTG. Next, 5-kb *Hin*dIII fragments were gel excised from pBS and ligated back into the pCCI, thus generating haplotypes *APOA5*3* and *APOA5*4.* We then cloned these sequences into the pMP8NEB delta lacZ vector [31] (a kind gift from Sarah K. Bronson) using *Not*I. To verify there were no mistakes in orientation and nucleotide sequence of *APOA5* and the haplotype-determining alleles, we analyzed these vectors using restriction-enzyme-based analysis and sequencing.

Hprt targeting

All four *APOA5* haplotypes within the pMP8NEB delta lacZ vectors were linearized with *Not*I restriction enzyme and electroporated into an E14TG2a *Hprt*-deficient 129 ES cell line [32]. Cells were grown on murine embryonic fibroblasts in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone), L-glutamine (Gibco), nonessential amino acids (Gibco), Hepes (Gibco), β-mercaptoethanol (Sigma), penicillin/streptomycin (Gibco), and ESGRO (Chemicon). Positive colonies were selected using HAT medium and were screened by PCR using primers that spanned the mouse *Hprt* gene and human AV gene junction. Sequences of primers were mHPRT F, 5′-TGAGTTCCTGCATTGAGCAACTGA-3′, and hApoAV R, 5′-TGAGATGCAGAGGGGACACTTGG-3′. For Southern blot 10 μg of genomic DNA was digested with *Sca*I and the probe was synthesized using the following PCR primer pair: 5′-TTATGGTACTGGCAGGGAGATTAGG-3′ and 5′-CAGAACATCTTGAAACCCAGCATCC-3′. The resultant male chimeras were bred to C57BL/6J females, and heterozygous females generated from these crosses were mated with C57BL/6J males to generate transgene-positive hemizygous males.

Triglyceride measurements

All animals were fed standard chow diet and were analyzed between 2 and 4 months of age. Blood samples were collected after a 5-h fast by retro-orbital bleeding using heparinized microhematocrit tubes. Total triglyceride concentrations were measured using enzymatic methods (Sigma 337-A) in 96-well plates using a Spectramax 250 (Molecular Devices).

APOA5 measurements

A pool of two monoclonal anti-human APOAV antibodies raised in mice using recombinant protein was used at 10 μg/ml in 0.1 M PBS, pH 7.2, to coat the wells of the microtiter plates at room temperature overnight. The wells were washed twice with 0.1 M PBS and then saturated with 3% BSA/PBS for 1 h at 37°C. For quantitation, a pool of human plasma was calibrated and titrated using ApoAV recombinant protein as a primary standard. Then, the pool of plasma was used for the calibration curve. All dilutions were done in the blocking buffer (1% BSA/PBS). One hundred microliters of the antigen solution was added to the wells and incubated for 2 h at room temperature. The wells were washed four times with PBS followed by the addition of an anti-ApoAV polyclonal antibody (1:400) produced in goat using synthetic peptide and incubated for 2 h at 37°C. Unbound antibody was removed by washing four times, and 100 μl of goat anti-rabbit IgG-biotin conjugate diluted 1:20,000 (Sigma) was added to each well. The plates were incubated for 1 h at 37°C and washed several times. Following incubation with the biotinylated antibody, a solution of streptavidin-alkaline phosphatase (1:500; Sigma) was added for 30 min at 37°C. Then, detection using *p*-nitrophenyl phosphate substrate (Sigma) was performed in the dark at room temperature and the absorbance at 405 nm was measured using a microplate photometer (Dynex Technologies).

Real-time quantitative PCR

Total RNA was extracted from liver tissue of adult mice using TRIzol reagent (Invitrogen). Following reverse transcription with the SuperScript First-Strand Synthesis System (Invitrogen), real-time PCR was performed using human *APOA5* gene-specific primers (5′-TCCTTGTCTGGGGAGCCTTGG-3′, and 5′-CAACAGGCCACTTTCAAGGACTGA-3′), QuantumRNA Universal 18S (Ambion), and the SYBR Green PCR Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). All procedures and calculations were carried out according to the manufacturer's recommendations.

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